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HISTOSPECIFIC ACETYLCHOLINESTERASE DEVELOPMENT IN QUARTER ASCIDIAN EMBRYOS DERIVED FROM EACH BLASTOMERE PAIR OF THE EIGHT-CELL STAGE

TAKUYA DENO, HIROKI NISHIDA, AND NORIYUKI SATOH

Department of Zoology, Kyoto University, Kyoto 606, and Asamushi Marine Biological Station of Tohoku University, Aomori 039-34, Japan

Abstract

Recent cell lineage studies of ascidian embryos have shown that muscle cells of the larval tail are derived not only from the B4.1-cell pair of 8-cell embryos, as was formerly believed, but also from the b4.2- and A4.1-cell pairs. Therefore, we re-examined the developmental autonomy of blastomere pairs in 8-cell ascidian embryos. The four blastomere-pairs (a4.2, b4.2, A4.1, and B4.1) were isolated from the 8-cell embryos of *Ciona intestinalis* and *Halocynthia roretzi* and allowed to develop into quarter embryos. More than 80% of the B4.1 quarter embryos of both species produced histochemically detectable, putative muscle-specific acetylcholinesterase (AChE). About 10% of the *Ciona* b4.2 quarter embryos and 1% of the *Halocynthia* b4.2 quarter embryos showed AChE activity. About 2% of the *Halocynthia* A4.1 quarter embryos developed AChE activity, but none of the *Ciona* A4.1 quarter embryos showed AChE activity. Although the frequency of the b4.2 or A4.1 quarter embryos with AChE activity was relatively low, these results indicate that not only isolated B4.1 blastomeres but also isolated b4.2 or A4.1 blastomeres could produce AChE independently from the interaction with progeny cells of the other pairs. In addition, about 3–4% of the a4.2 quarter embryos of both species produced AChE. This activity, found in cells thought not to contribute to the muscle cell lineage, may be due to the expression of AChE activity in the larval brain of *Ciona* and the larval brain and pharynx of *Halocynthia*.

Introduction

Descriptive and experimental studies have demonstrated that the selection of different developmental pathways in ascidian embryos is not mediated by a stable intrinsic nuclear lineage but by cytoplasmic determinants localized in predetermined regions of the egg (Conklin, 1905a, b; Reverberi and Minganti, 1946; Whittaker, 1973, 1980, 1982; Tung *et al.*, 1977; Deno and Satoh, 1984; Deno *et al.*, 1984). The cytoplasmic determinants are thought to be segregated by clevage into certain lineage cells, where they bring about the activation of genes responsible for tissue-specific enzyme development (see Davidson, 1976; Whittaker, 1979; Jeffery *et al.*, 1984 for reviews).

One line of evidence for the segregated cytoplasmic determinants is the capacity of isolated blastomeres to differentiate autonomously according to the developmental fates predicted by their cell lineages (see Reverberi, 1971; Whittaker, 1979 for reviews). According to the cell lineages devised by Conklin (1905a) and Ortolani (1955), muscle cells of the larval tail originate from the B4.1 pair of blastomeres (the posterior vegetal blastomeres) of the 8-cell stage (Fig 1). Autonomous develop-

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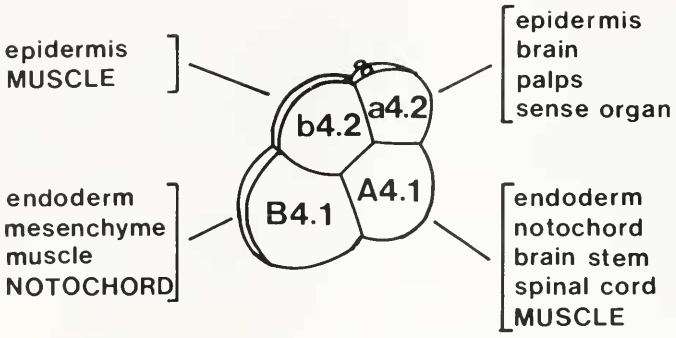

FIGURE 1. A diagram illustrating the nomenclature of blastomeres of the 8-cell-stage ascidian embryos according to Conklin (1905a), and also listing the derivatives from each blastomere pair. The derivatives shown with small letters are based on previous studies (Conklin, 1905a; Ortolani, 1955) while those with capitals are those obtained in our recent study (Nishida and Satoh, 1983).

ment of a putative muscle-specific enzyme, acetylcholinesterase (AChE), and of myofibrils has been shown in partial ascidian embryos derived from isolated B4.1 pairs (Whittaker *et al.*, 1977; Crowther and Whittaker, 1983). Recent analyses of cell lineages in ascidian embryos, however, have demonstrated that muscle cells are derived not only from the B4.1-cell pair, as was formerly believed, but also from both the A4.1- (the anterior vegetal blastomeres) and b4.2-cell pairs (the posterior animal blastomeres) (Fig. 1; Nishida and Satoh, 1983; Zaloker and Sardet, 1984). In a previous study, we isolated the B4.1-cell pairs from 8-cell *Ciona* embryos and showed that AChE development as well as myofibril differentiation took place in partial embryos originating not only from isolated B4.1 pairs, but also from 8-cell embryos lacking B4.1 progeny cells (Deno *et al.*, 1984). The goal of the present study was to determine the ability of quarter embryos derived from the isolated B4.1, A4.1, b4.2, and a4.2 pairs to express AChE activity.

MATERIALS AND METHODS

Animals, gametes, and embryos

Eggs of the ascidians *Ciona intestinalis* (L.) and *Halocynthia roretzi* (Drashe) were used in this study. *C. intestinalis* adults were collected at Takahama, Wakasa Bay, Japan and maintained in temperature-controlled aquaria (18°C) under constant light to induce oocyte maturation. Eggs were removed surgically from the gonoducts and fertilized with a dilute suspension of sperm of other individuals. Fertilized eggs were reared in filtered sea water at 18°C. Under these conditions they reached the 8-cell stage about 2 h after fertilization and hatched at about 17 h of development. *H. roretzi* adults were collected in Mutsu Bay, Aomori, Japan and maintained in aquaria of the Marine Biological Station of Asamushi. Naturally spawned eggs were fertilized by mixing sperm suspensions of different animals, and reared in filtered sea water at 13°C. At this temperature, they developed to the 8-cell stage about 3.5 h after fertilization and hatched at about 34 h of development.

Blastomere isolations

Each experiment was carried out in a room in which the temperature was 18°C for *Ciona* embryos and 13°C for *Halocynthia* embryos. Fertilized eggs of both species

were dechorionated with sharpened tungsten needles between 10 and 30 min after fertilization. Dechorionated eggs were cultured to the 8-cell stage in 0.9% agar coated Falcon petri dishes. Only 8-cell embryos of normal appearance were used for the experiments. The four cell-pairs of the 8-cell embryos (*i.e.*, a4.2 + a4.2, b4.2 + b4.2, A4.1 + A4.1, and B4.1 + B4.1) were separated with a glass needle under a dissecting microscope (Fig. 2). The location of polar bodies, the configurations of the blastomeres, and the distribution of pigments were used as landmarks for orientation of the embryos. Isolated blastomeres were cultured separately in 0.9% agar coated Falcon 24-well multiwells. For culture of dechorionated embryos and isolated blastomeres, Millipore-filtered (pore size, 0.2 μm) sea water containing 50 μg/ml streptomycin sulfate was used. Dechorionated control embryos and isolates were reared until the appropriate developmental stages, and then fixed for histochemical examinations.

Enzyme histochemistry

AChE is thought to be a tissue-specific enzyme of muscle cells in the tail of developing ascidian embryos (Durante, 1956; Meedel and Whittaker, 1979). The enzyme activity was detected histochemically in dechorionated whole embryos as well as in partial embryos by the "direct-coloring" thiocholine method of Karnovsky and Roots (1964) using acetylthiocholine iodide as a substrate. In the case of *C. intestinalis* the embryos were first cooled at 4°C then fixed with cold (4°C) 80% ethanol for only 30 s as described previously (Deno *et al.*, 1984), whereas *Halocynthia* embryos were fixed with cold (4°C) 5% formalin sea water for 30 min. It has been revealed that the enzyme activity detected by the present histochemical technique is attributable to the presence of AChE and not to pseudocholinesterase (Fromson and Whittaker, 1970; Meedel and Whittaker, 1979; Satoh, 1979). Histochemically stained embryos were dehydrated in a graded series of ethanol solutions, cleared in xylene, and mounted in balsam for microscopic examination and photomicrography.

Results

Acetylcholinesterase development in control embryos

Dechorionated eggs were allowed to develop in microwells for the same length of time as that of partial embryos. These control embryos of both species, however, did not always develop to the normal tailbud stage. Although the reason is obscure, a similar inclination was noticed in previous studies (Whittaker, 1982; Deno *et al.*,

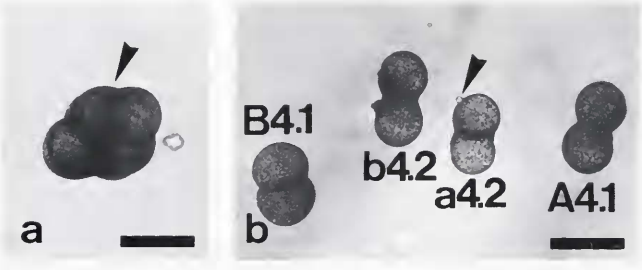

FIGURE 2. Photomicrographs demonstrating blastomere isolation. (a) Dechorionated 8-cell-stage *Ciona* embryo viewed from the left side. (b) Isolated blastomere pairs from the 8-cell-stage embryo. Arrowheads indicate the polar bodies, which mark the animal pole of the egg. Scale bar, 50 μm.

1984). In this study, 64% (234/264) of the dechorionated 8-cell-stage embryos of *C. intestinalis* formed morphologically normal tailbud stages. However, almost all of these tailbud stages which had developed for more than 8 h produced AChE activity in tail muscle cells (Fig. 3a). In addition to the enzyme activity localized in muscle cells, some control embryos examined at 13.5 h of development showed the enzyme activity in a strand-like structure in the dorsal region of the head, as did almost all of the control embryos which had developed for more than 15 h (Fig. 3b). Furthermore, a spot of AChE activity appeared in a region posterior to melanocytes of all 17-h embryos examined (Fig. 3c). This spot appeared to be attached to the strand-like structure (Fig. 3c). From its location, the spot of AChE activity seems to be a part of the central nervous system, which may presumably be the "adult brain," as has been pointed out by Meedel and Whittaker (1979).

More than half of dechorionated 8-cell embryos of *H. roretzi* failed to complete neural-tube formation, resulting in morphologically abnormal embryos. However, these abnormal embryos, as well as morphologically normal tailbud stages which had developed for more than 14 h, showed AChE activity in tail muscle cells (Fig. 4a), suggesting a normal production of AChE in spite of the failure of normal

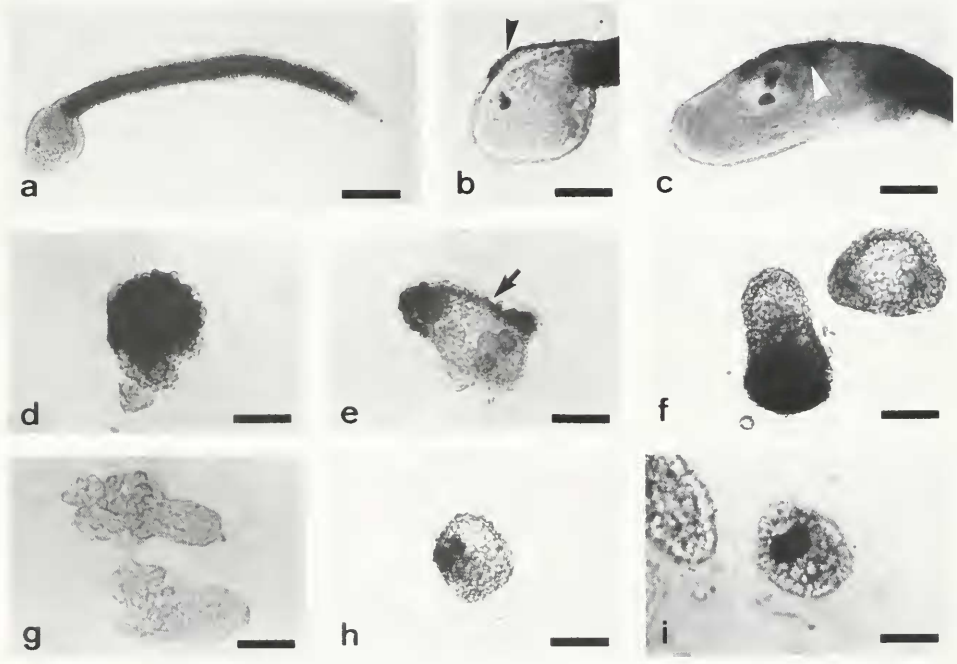

FIGURE 3. Histochemical localization of AChE activity in *C. intestinalis* whole and quarter embryos of various ages. (a) Tailbud-stage embryo developed from dechorionated whole egg (13 h of development). AChE activity developed only in the muscle cells of the tail. (b, c) Head regions of dechorionated whole embryos after 13.5 h (b) and 19 h (c) of development, respectively. Arrowhead in (b) indicates a strand-like structure with AChE activity and arrowhead in (c) indicates "adult brain" with the enzyme activity. (d) B4.1 quarter embryo showing AChE activity (13.5 h of development). (e, f) b4.2 quarter embryos with AChE activity; (e) 13.5 h, (f) 19 h. A right quarter embryo in (f) does not show the enzyme activity. Arrow indicates a strand-like ectodermal structure with the enzyme activity. (g) A4.1 quarter embryos (13.5 h of development). They did not show AChE activity. (h, i) a4.2 quarter embryos at 19 h (h) and 22 h (i) of development, respectively. A patch of cells clearly show the enzyme activity. Scale bars, 50 μm in (a) and 25 μm in (b–i).

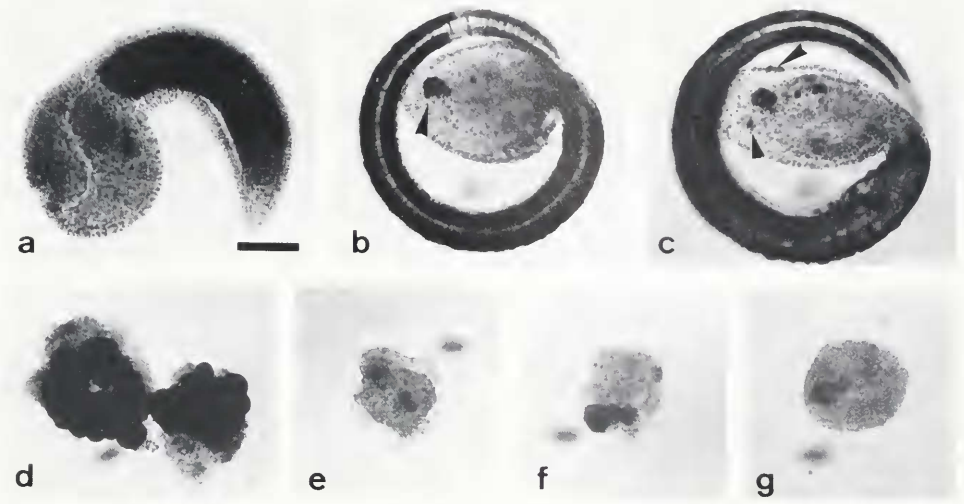

FIGURE 4. Histochemical localization of AChE activity in *H. roretzi* whole and quarter embryo of various ages. (a, b, c) Dechorionated whole embryos after 22 h (a), 28 h (b), and 32 h (c) of development, respectively. In (a) muscle cells of the caudal tip region do not produce AChE, but in (b) and (c) they show the enzyme activity. Arrowhead in (b) indicates the primordial pharynx with AChE activity and arrowheads in (c) indicate small spots with the enzyme activity. (d) B4.1 quarter embryos with AChE activity (30 h of development). (e) b4.2 quarter embryo at 30 h of development. A few cells show the enzyme activity. (f) A4.1 quarter embryo with the enzyme activity (30 h of development). (g) a4.2 quarter embryos developing AChE activity in a few cells (46 h of development). Scale bar, 100 μm.

morphogenesis. At this time, however, muscle cells of the caudal tip of the tail did not show the enzyme activity; AChE activity in this region first appeared in 24-h embryos (Fig. 4b). Like *Ciona* embryos, normal *Halocynthia* embryos also produced AChE in tissues other than the tail muscle cells. AChE activity was noticed in the region between the sensory vesicle and the papillae in 24-h embryos (Fig. 4b). This structure is in the region of the primordial pharynx. In addition, 32-h embryos produced AChE in several other small spots near both dorsal and ventral sides of the primordial pharynx region (Fig. 4c).

Acetylcholinesterase development in quarter embryos

In this report, partial embryos derived from isolated a4.2-, b4.2-, A4.1-, and B4.1-blastomere pairs are designated for convenience as a4.2, b4.2, A4.1, and B4.1 quarter embryos, respectively. AChE activity in these quarter embryos is summarized in Table I (*C. intestinalis*) and in Table II (*H. roretzi*).

The B4.1 quarter embryos. According to recent cell lineage study (Nishida and Satoh, 1983), among the 36 (*C. intestinalis*) or 42 (*H. roretzi*) muscle cells of the larval tail, 28 cells located in the anterior and middle parts of the tail originate from the B4.1 pair of blastomeres. In *C. intestinalis* isolated B4.1-pairs usually developed to raspberry-shaped partial embryos (Fig. 3d). B4.1 quarter embryos which developed for more than 12 h (beyond the normal time of AChE synthesis) produced AChE activity (Fig. 3d) at a high frequency (92% on an average; Table I). The B4.1 quarter embryos of *H. roretzi* which had developed for more than 22 h also became raspberry-like cell aggregates (Fig. 4d). They also frequently developed AChE activity (81% on an average; Table II; Fig. 4d).

TABLE I

Development of histochemically detectable acetylcholinesterase activity in quarter Ciona intestinalis *embryos*

Origin of quarter embryos	Time (h) of development No. of batches	No. of embryos with acetylcholinesterase activity						
		12	13.5	15	17	19	22	Total
		3	2	2	1	1	1	10
B4.1-cell pair		53/59 (90%)	76/78 (97%)	42/48 (88%)	20/23 (87%)	14/14 (100%)	29/32 (91%)	234/254 (92%)
b4.2-cell pair		0/82 (0%)	3/51 (6%)	6/44 (14%)	5/42 (12%)	3/23 (13%)	4/37 (11%)	21/279 (8%)
A4.1-cell pair		0/78 (0%)	0/56 (0%)	0/38 (0%)	0/30 (0%)	0/14 (0%)	0/33 (0%)	0/249 (0%)
a4.2-cell pair		0/82 (0%)	0/69 (0%)	2/45 (4%)	1/30 (3%)	2/23 (9%)	4/38 (11%)	9/287 (3%)

TABLE II

Development of histochemically detectable acetylcholinesterase activity in quarter Halocynthia roretzi *embryos*

Origin of quarter embryos	No. of embryos with acetylcholinesterase activity				
	Time (h) of development	22	26	30	46	Total
	No. of batches	4	3	5	5	17
B4.1-cell pair		147/242 (61%)	307/357 (86%)	200/208 (96%)	317/389 (82%)	971/1196 (81%)
b4.2-cell pair		0/252 (0%)	2/361 (1%)	2/187 (1%)	10/313 (3%)	14/1113 (1%)
A4.1-cell pair		2/215 (1%)	3/314 (1%)	2/218 (1%)	13/352 (4%)	20/1099 (2%)
a4.2-cell pair		0/268 (0%)	5/338 (2%)	3/201 (2%)	35/327 (11%)	43/1134 (4%)

The b4.2 quarter embryos. A recent cell lineage study has reported that 4 (*C. intestinalis*) or 10 (*H. roretzi*) muscle cells of the caudal tip region are derived from the b4.2-cell pair (Nishida and Satoh, 1983). The b4.2 quarter embryos of *C. intestinalis* developed into a blastula-like structure with a cell mass in the cavity (Figs. 3e, f). AChE activity was not found in the b4.2 quarter embryos examined at 12 h of development (0/82; Table I). However, localized enzyme activity began to appear in some of the b4.2 quarter embryos at 13.5 h (3/51, 6%; Table I), and an average of 12% of the b4.2 quarter embryos which had developed for more than 15 h showed AChE activity (Figs. 3e, f; Table I). Most of the b4.2 quarter embryos that developed AChE activity showed it only in a small number of cells in the interior portion of the blastula-like structure (Fig. 3f), but some embryos developed the enzyme activity not only in these cells but also in the cell of a strand-like ectodermal structure (Fig. 3e).

The b4.2 quarter embryos of *H. roretzi* also developed into blastula-like structures with a cell-mass in the cavity (Fig. 4e). No AChE activity was found in the b4.2 quarter embryos examined at 22 h of development (0/252; Table II). The b4.2 quarter embryos which had developed for more than 26 h, however, produced localized enzyme activity, although the frequency was very low (14/861, 1.6%; Table II; Fig. 4e). In contrast to *Ciona* b4.2 quarter embryos, a strand-like structure with the enzyme activity did not appear in *Halocynthia* b4.2 quarter embryos.

The A4.1 quarter embryos. Four muscle cells of the posterior part of the larval tail are the descendants of the A4.1-cell pair. Isolated A4.1-cell pairs of *C. intestinalis* gave rise to gourd-shaped partial embryos. However, no AChE activity was detected in the A4.1 quarter embryos examined at times between 12 and 22 h after fertilization (0/249; Table I; Fig. 3g). The A4.1 quarter embryos of *H. roretzi* became raspberry-shaped structures similar to those produced by the B4.1 quarter embryos. Although the percentage of the positive embryos was low (2% on the average; Table II), the A4.1 quarter embryos did develop enzyme activity in some of their cells (Fig. 4f).

The a4.2 quarter embryos. According to all the current ascidian cell lineage studies the a4.2-cell pair gives rise to cells of the epidermis, brain, and sensory organs, but not to tail muscle cells (Fig. 1). The a4.2 quarter embryos of *C. intestinalis,* similar to the b4.2 quarter embryos, developed into blastula-like structures with clumps of tissue in the cavity (Figs. 3h, i). Unexpectedly, some of the a4.2 quarter embryos also developed AChE. The enzyme activity first appeared at 15 h of development (4%; Table I), and the proportion of quarter embryos with enzyme activity increased to about 10% by 19 h of development (Table I). The distribution of AChE activity in the a4.2 quarter embryos was not spread but localized in particular cells (Figs. 3h, i).

Blastula-like a4.2 quarter embryos of *H. roretzi* also produced AChE activity, which first appeared after 26 h of development (Fig. 4g). About 11% of the 46-h, a4.2 quarter embryos also showed the enzyme activity (Table II).

Discussion

More than two-thirds of muscle cells in the larval tail are descendants of the B4.1-cell pair at the 8-cell stage. As clearly shown in this study, if the B4.1 blastomeres are isolated and allowed to develop into quarter embryos, these partial embryos produce AChE at a high frequency (more than 90% in *Ciona* and about 80% in *Halocynthia*). This result confirms previous studies (Whittaker *et al.*, 1977; Whittaker, 1982; Deno *et al.*, 1984).

The b4.2 quarter embryos of both species as well as *Halocynthia* A4.1 quarter embryos also showed the enzyme activity. In *Ciona* b4.2 quarter embryos AChE activity was found in a strand-like epidermal structure in addition to some cells in the interior of the blastula-like structures that formed. According to the most recent cell lineage study (Nishida and Satoh, 1983) a strand of epidermal cells at the dorsal head region of tailbud embryos is derived from the b4.2-cell pair, and as shown in this study, this structure can develop AChE. Therefore, the strand-like structure exhibiting AChE activity in the *Ciona* b4.2 quarter embryos may be correlated with the epidermal structure of normal embryos.

The frequency of AChE development in b4.2 or A4.1 quarter embryos was very low, particularly in A4.1 quarter embryos. The reason for this is obscure, but blastomere isolation could disturb the normal division pattern of the isolated cells, causing abnormal segregation of the cytoplasmic determinants responsible for AChE development. However, the possibility that induction phenomena between blastomeres are involved in AChE development in b4.2 or A4.1 quarter embryos cannot be ruled out (Pucci-Minafra and Ortolani, 1968). So far, several blastomere isolation experiments in ascidian embryos have demonstrated muscle cell development in partial embryos lacking the progeny cells of B4.1 blastomeres (Von Ubisch, 1939; Reverberi and Minganti, 1946, 1947). In the previous study, for instance, we showed that more than 85% of *Ciona* a4.2 + b4.2 + A4.1 partial embryos developed AChE (Deno *et al.*, 1984). However, we know of no report of muscle development in the b4.2 or A4.1 quarter embryos. If cleavage-stage ascidian embryos are permanently arrested with cytochalasin B, an inhibitor of cytokinesis, the arrested embryos produced AChE in muscle lineage blastomeres (Whittaker, 1973a; Satoh, 1979). However, the blastomeres which developed enzyme activity were only the B4.1-line muscle lineage cells; the b4.2- and A4.1-line muscle lineage cells did not produce the enzyme. This phenomenon may be related to the low frequency of b4.2 or A4.1 quarter embryos that show the enzyme activity. In any event the present study clearly shows that, in addition to the B4.1 quarter embryos, the b4.2 or A4.1 quarter

embryos also produced AChE, although the frequency of the embryos with the enzyme activity is low. The results also support the recent cell lineage study suggesting that muscle lineage cells of the ascidian embryos arise from more than one line of founder cells.

Unexpectedly, about 3–4% of the a4.2 quarter embryos of both species also developed AChE. As shown in a previous study (Meedel and Whittaker, 1979) as well as in this study, *Ciona* embryos and larvae produce AChE in the brain, whereas *Halocynthia* embryos produce AChE in the brain and/or the pharynx region, in addition to the tail muscle cells. The brain and pharynx originate from the a4.2-cell pair at the 8-cell stage (Nishida and Satoh, 1983). In each species the time of the first appearance of AChE activity in the a4.2 quarter embryos almost coincided with that at which normal embryos differentiated the enzyme activity in these tissues. Therefore, it is unlikely that AChE development in the a4.2 quarter embryos is related to muscle differentiation. Instead it is probably associated with the differentiation of the brain cells (*Ciona* and *Halocynthia* embryos) or with pharynx cells (*Halocynthia* embryos).

This study suggests a way to improve the biological assay system for identifying and purifying the cytoplasmic determinant(s) responsible for AChE development. Recently, we have transplanted cytoplasm from B4.1 blastomeres of 8-cell *Halocynthia* embryos into the A4.1 blastomeres of another embryo by microinjection (Deno and Satoh, 1984). When the host 8-cell embryos were then arrested with cytochalasin B, a few of them developed AChE activity in the A4.1 cells in addition to the B4.1 cells. At the time these experiments were being done this result suggested a possible assay system for isolation and characterization of cytoplasmic determinants. The recent cell lineage study, however, revealed that some of the tail muscle cells are derived from the A4.1 blastomeres, and suggest that a better assay system might be developed by transplanting cytoplasm from the B4.1 cells into the a4.2 cells (Nishida and Satoh, 1983). However, as clearly shown in this study, a finite number of the a4.2 blastomere progeny also produce AChE without differentiating into muscle. Therefore, the a4.2 cells are not necessarily the most desirable hosts for the assay system. Instead, cytoplasm from B4.1 cells or from isolated yellow crescents (Jeffery *et al.*, 1984) should be injected into an isolated A4.1-cell pair and be allowed to develop into a quarter embryo. If five or more progeny cells of the transplanted A4.1-cell pair showed the enzyme activity, this could be used as a potential biological assay for cytoplasmic determinants.

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